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Identification, production and use of new staphylokinase derivatives with reduced immunogenicity

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IDENTIFICATION, PRODUCTION AND USE OF NEW STAPHYLOKINASE DERIVATIVES WITH REDUCED IMMUNOGENICITY

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This invention relates to new staphylokinase derivatives with reduced immunogenicity, their identification, production and use in the treatment of arterial thrombosis and the preparation of a pharmaceutical composition for treating arterial thrombosis. More in particular it relates to the use of engineered staphylokinase derivatives for the preparation of a pharmaceutical composition for treating myocardial infarction.

Staphylokinase, a protein produced by certain strains of *Staphylococcus aureus*, which was shown to have profibrinolytic properties more than 4 decades ago (1,2) appears to constitute a potent thrombolytic agent in patients with acute myocardial infarction (3,4). The staphylokinase gene has been cloned from the bacteriophages *sak*øC (5) and *sak*42D (6) as well as from the genomic DNA (*sak*STAR) of a lysogenic *Staphylococcus aureus* strain (7). The staphylokinase gene encodes a protein of 163 amino acids, with amino acid 28 corresponding to the NH_2-terminal residue of full length mature staphylokinase (6,8,9). The mature protein sequence of the wild-type variant SakSTAR (9) is represented in Figure 1. Only four nucleotide differences were found in the coding regions of the *sak*ϕC, *sak*42D and *sak*STAR genes, one of which constituted a silent mutation (6,8,9).

In a plasma milieu, staphylokinase is able to dissolve fibrin clots without associated fibrinogen degradation (10-12). This fibrin-specificity of staphylokinase is the result of reduced inhibition by α_2-antiplasmin of plasmin.staphylokinase complex bound to fibrin, recycling of staphylokinase from the plasmin.staphylokinase complex following inhibition by α_2-antiplasmin, and prevention of the conversion of circulating plasminogen.staphylokinase to plasmin.staphylokinase by α_2-antiplasmin (13-15). In addition staphylokinase has a weak affinity for circulating but a high affinity for fibrin-bound plasminogen (16) and staphylokinase requires NH_2-terminal processing by plasmin to display its plasminogen activating potential (17). In several experimental animal models, staphylokinase appears to be equipotent to streptokinase for the dissolution of whole blood or plasma clots, but significantly more potent for the dissolution of platelet-rich or retracted thrombi (18,19).

conditions suitable for expressing the DNA fragment and purifying the expressed staphylokinase derivative to homogeneity; preferably the DNA fragment is a 453 bp *Eco*RI-*Hin*dIII fragment of the plasmid *pMEX602sakB* (22,23), the *in vitro* site-directed mutagenesis is preferably performed by spliced overlap extension polymerase chain reaction with Vent DNA polymerase (New England Biolabs) or Taq polymerase (Boehringer Mannheim) and with available or generated wildtype *sakSTAR* or *sakSTAR* variants as template (24).

The invention also relates to pharmaceutical compositions comprising at least one of the staphylokinase derivatives according to the invention together with a suitable excipient, for treatment of arterial thrombosis. Pharmaceutical compositions, containing less immunogenic staphylokinase variants as the active ingredient, for treating arterial thrombosis in human or veterinary practice may take the form of powders or solutions and may be used for intravenous, intraarterial or parenteral administration. Such compositions may be prepared by combining (e.g. mixing, dissolving etc.) the active compound with pharmaceutically acceptable excipients of neutral character (such as aqueous or non-aqueous solvents, stabilizers, emulsifiers, detergents, additives), and further, if necessary with dyes.

Furthermore the invention relates to the use of the staphylokinase derivatives for the treatment of arterial thrombosis, in particular myocardial infarction, and to the use of staphylokinase derivatives for the preparation of a pharmaceutical composition for the treatment of arterial thrombosis, in particular myocardial infarction.

In the above and the following the terms "derivatives", "mutants" and "variants" are used interchangeably.

The present invention will be demonstrated in more detail in the following examples, that are however not intended to be limiting to the scope of the invention. Based on the present invention other variants and improvements will be obvious for the person skilled in the art. Thus random mutagenesis is likely to generate alternative mutants with reduced immunogenicity and possibly increased functional activity, whereas deletions or substitution with other amino acids may yield additional variants with reduced immunogenicity.

In the tables the column indicated with "Variant" states the various staphylokinase derivatives which are identified by listing between brackets the substituted amino acids in single letter symbols followed by their position number in the mature staphylokinase sequence and by the substituting amino acids in single letter symbol; the column "Exp." indicates expression levels in mg/L, and the column "Spec. Act." indicates the specific activity in Home Units as defined in example 2. Indications "17G11", "26A2" etc. refer to monoclonal antibodies binding to the indicated epitopes I, II and III (22). Epitope I is recognized by the antibody cluster 17G11, 26A2, 30A2, 2B12 and 3G10, whereas epitope II is recognized by the antibody cluster 18F12, 14H5, 28H4, 32B2 and 7F10, and epitope III by the antibody cluster 7H11, 25E1, 40C8, 24C4 and 1A10. Human plasma "Pool" indicates a plasma pool from initially 16 and subsequently 10 patients immunized by treatment with SakSTAR, "Subpool B" indicates a plasma pool from three patients that absorbed less than 50% of the induced antibodies with SakSTAR(K35A,E38A,K74A,E75A,R77A) and "Subpool C" indicates a plasma pool from 3 patients that absorbed >90% of the induced antibodies with SakSTAR(K35A,E38A,K74A,E75A,R77A) (22). In tables 6, 7 and 8 an additional pool of plasma from 40 patients immunized by treatment with SakSTAR (Pool 40) was also used.

EXAMPLE 2

Construction, epitope mapping with murine monoclonal antibodies and absorption with pooled plasma of immunized patients, of "alanine-to-wild-type" reversal variants of "charged-cluster-to-alanine" mutants of staphylokinase

As stated above, wild-type staphylokinase (SakSTAR variant (9)) contains three non-overlapping immunodominant epitopes, two of which can be eliminated by specific site-directed substitution of clusters of two (K35A,E38A or E80A,D82A) or three (K74A,E75A,R77A) charged amino acids with Ala (22). The combination mutants SakSTAR(K35A,E38A,K74A,E75A,R77A) in which Lys35, Glu38, Lys74, Glu75 and Arg77, and SakSTAR(K74A,E75A,R77A,E80A,D82A) in which Lys74, Glu75, Arg77, Glu80 and Asp82 were substituted with Ala (previously identified as SakSTAR.M3.8 and

procedure. DNA sequencing was performed using the dideoxy chain termination reaction method and the Automated Laser fluorescent A.L.F.™ (Pharmacia). The chromogenic substrate (S2403) L-Pyroglutamyl-L-phenylalanyl-L-lysine-p-nitroanaline hydrochloride was purchased from Chromogenix (Belgium). ^{125}I-labeled fibrinogen was purchased from Amersham (UK). All other methods used in the present example have been previously described (22,27).

Construction of expression plasmids

The plasmids encoding SakSTAR(K35A,E38A,K74A,E75A), SakSTAR(E38A,E75A,R77A), SakSTAR(E38A,E75A), SakSTAR(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E80A), SakSTAR(D82A), SakSTAR(E75A,D82A), SakSTAR(K74A) and SakSTAR(E75A) were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24), using Vent DNA polymerase (New England Biolabs, Leusden, The Netherlands), and available or generated *sakSTAR* variants as template. Two fragments were amplified by PCR, the first one starting from the 5' end of the *staphylokinase* gene with primer 5'-CAGGAAACAGAATTCAGGAG-3' to the region to be mutagenized (forward primer), the second one from the same region (backward primer) to the 3' end of the *staphylokinase* gene with primer 5'-CAAAACAGCCAAGCTTCATTCATTCAGC-3'. The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled together in a new primerless PCR using Taq polymerase (Boehringer Mannheim). After 7 cycles (1 min at 94°C, 1 min at 70°C), the extended product was reamplified by adding the 5' and 3' end primers (see above) to the PCR reaction and by cycling 25 times (1 min at 94°C, 1 min 55°C, 1 min at 72°C). The final product was purified, digested with EcoRI and HindIII and cloned into the corresponding sites of *pMEX602sakB*.

The plasmid encoding SakSTAR(E38A,K74A,E75A,R77A) was assembled by digestion of *pMEX602sakB* and *pMEX.SakSTAR(K35A,E38A,K74A,E75A,R77A)* with Bpm I which cuts between the codons for K35 and E38 of SakSTAR, and ligation of the required fragments. The plasmid encoding SakSTAR(K35A,K74A,E75A,R77A) was constructed by digestion of

Cleared cell lysates containing the SakSTAR variants were subjected to chromatography on a 1.6 x 6 cm column of SP-Sephadex, followed by chromatography on a 1.6 x 5 cm column of Q-Sepharose [variants SakSTAR(E38A,K74A,E75A,R77A), SakSTAR(K35A,K74A,E75A, R77A), SakSTAR(K35A,E38A,E75A,R77A), SakSTAR(K35A,E38A,K74A,R77A) and SakSTAR(K35A,E38A,K74A,E75A)] or by chromatography on a 1.6 x 6 cm column of phenyl-Sepharose [variants SakSTAR(E35A,E38A,R77A), SakSTAR(E38A,E75A), SakSTAR(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(K74A), SakSTAR(E75A), SakSTAR(E80A), SakSTAR(D82A) and SakSTAR(E75A,D82A)]. The SakSTAR containing fractions, localized by SDS-gel electrophoresis, were pooled for further analysis.

Physicochemical and biochemical analysis

Protein concentrations were determined according to Bradford (29). The specific activities of SakSTAR solutions were determined with a chromogenic substrate assay carried out in microtiter plates using a mixture of 80 µL SakSTAR solution and 100 µL Glu-plasminogen solution prepared as described elsewhere (30) (final concentration 0.5 µmol/L). After incubation for 30 min at 37°C, generated plasmin was quantitated by addition of 20 µL S2403 (final concentration 1 mmol/L) and measurement of the absorption at 405 nm. The activity was expressed in home units (HU) by comparison with an in-house standard (lot STAN5) which was assigned an activity of 100,000 HU (100 kHU) per mg protein as determined by amino acid composition (7). SDS-PAGE was performed with the Phast System™ (Pharmacia, Uppsala, Sweden) using 10-15% gradient gels and Coomassie Brilliant blue staining. Reduction of the samples was performed by heating at 100°C for 3 min in the presence of 1% SDS and 1% dithioerythritol. The specific activities of the different SakSTAR mutants determined with the chromogenic substrate assay are summarized in Table 1.

clot lysis in 20 min was determined. The neutralizing activity titer was determined as the difference between the test plasma and buffer values and was expressed in µg per mL test plasma. The results of the individual patients have been reported elsewhere (22). For the present invention, three plasma pools were made, one from 10 patients from whom sufficient residual plasma was available, one from three patients that absorbed less than 50% of the antibodies with SakSTAR(K35A,E38A,K74A,E75A,R77A) (Subpool B) and one from three patients that absorbed >90% of the antibodies with SakSTAR(K35A,E38A,K74A,E75A, R77A) (Subpool C).

These plasma pools were diluted (1/30 to 1/200) until their binding to SakSTAR substituted chips in the BIAcore instrument amounted to approximately 2000 RU. From this dilution a calibration curve for antibody binding was constructed using further serial two-fold dilutions. The plasma pools were absorbed for 10 min with 100 nmol/L of the SakSTAR variants, and residual binding to immobilized SakSTAR was determined. Residual binding was expressed in percent of unabsorbed plasma, using the calibration curve.

The results are summarized in Table 1. Whereas wild-type SakSTAR absorbed more than 95% of the binding antibodies from pooled plasma of the 10 patients, incomplete absorption (<60%) was observed with SakSTAR(K74A,E75A,R77A), SakSTAR(K35A,E38A,K74A, E75A,R77A), SakSTAR(E38A,K74A,E75A,R77A), SakSTAR(K35A,K74A,E75A,R77A), SakSTAR(K35A,E38A,K74A,R77A), SakSTAR(K35A,E38A,K74A,E75A), SakSTAR(K74A) and SakSTAR(K74A,E75A,R77A,E80A,D82A) but absorption was nearly complete with SakSTAR(K35A,E38A), SakSTAR(K35A,E38A,E75A,R77A), SakSTAR(E38A,E75A,R77A), SakSTAR(E38A,E75A), SakSTAR(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E75A), SakSTAR(E80A,D82A), SakSTAR(E80A), SakSTAR(D82A) and SakSTAR(E75A,D82A). These results, surprisingly, demonstrate that approximately 40% of the antibodies elicited in patients by treatment with wild-type SakSTAR depend on K74 for their binding (Table 1). Absorption with pooled plasma from 3 patients from which <50% of the antibodies were absorbed with SakSTAR(K35A,E38A,K74A,E75A,R77A) (Subpool B) confirmed the predominant role of K74 for antibody recognition. As expected, absorption with pooled plasma from 3 patients

Sepharose and on insolubilized SakSTAR, and elution of bound antibodies with 0.1 mol/L glycine-HCl, pH 2.8. The purity of the IgG preparation was confirmed by sodium dodecylsulfate polyacrylamide gel electrophoresis. In the IgM assays, titers defined as the plasma dilution giving an absorbancy at 492 nm equivalent to that of a 1/640 dilution of pooled plasma were determined and compared with the titer of baseline samples before treatment (median value 1/410, interquartile range 1/120-1/700).

Thrombolytic efficacy

Wild-type SakSTAR or the variants SakSTAR(K74A) or SakSTAR(K74A,E75A,R77A) were administered intra-arterially at or in the proximal end of the occlusive thrombus as a bolus of 2 mg followed by an infusion of 1 mg/hr (reduced overnight in some patients to 0.5 mg/hr) in groups of 6 to 12 patients with angiographically documented occlusion of a peripheral artery or bypass graft of less than 120 days duration. Patients were studied after giving informed consent, and the protocol was approved by the Human Studies Committee of the University of Leuven. Inclusion and exclusion criteria, conjunctive antithrombotic treatment (including continuous intravenous heparin) and the study protocol were essentially as previously described (22).

Relevant baseline characteristics of the individual patients are shown in Table 2. The majority of PAO were at the femoropopliteal level. Two iliac stent and 8 graft occlusions were included. Eight patients presented with incapacitating claudication, 5 with chronic ischemic rest pain, 7 with subacute ischemia and 7 with acute ischemia. One patient (POE) who had 2 years previously been treated with SakSTAR was included in the SakSTAR(K74A) group. This patient was not included in the statistical analyses.

Table 2 also summarizes the individual treatment and outcome. Intra-arterial infusion, at a dose of 6.0 to 25 mg and a duration of 4.0 to 23 hrs, induced complete recanalization in 24 patients and partial recanalization in 3. Complementary endovascular procedures (mainly PTA) were performed in 17 patients and complementary reconstructive vascular surgery following thrombolysis in 3. No patient underwent major amputation. Early recurrence of thrombosis after the end of the angiographic procedure occurred in 4 patients. Bleeding complications were absent or limited to mild to moderate hematoma formation at the

baseline and 1/640 at week 1 in patients treated with SakSTAR. Corresponding values at 2 weeks were 1/590 and 1/550 in patients given SakSTAR(K74A) and SakSTAR(K74A,E75A,R77A), not significantly different from 1/930 with SakSTAR (data not shown).

The antibodies induced by treatment with SakSTAR were completely absorbed by SakSTAR but incompletely by SakSTAR(K74A) and by SakSTAR(K74A,E75A,R77A) confirming the immunogenicity of the K74,E75,R77 epitope and the dominant role of K74 in the binding of antibodies directed against this epitope. The antibodies induced by treatment with SakSTAR(K74A) or SakSTAR(K74A,E75A,R77A) were completely absorbed by SakSTAR, by SakSTAR(K74A) and by SakSTAR(K74A,E75A,R77A), indicating that immunization was not due to neoepitopes generated by substitution of Lys74 with Ala, but to epitopes different from the K74,E75,R77 epitope.

Thus, this example illustrates that staphylokinase variants with *reduced* antibody induction but *intact* thrombolytic potency can be generated. The present experience in 26 patients treated with SakSTAR (n= 9), SakSTAR(K74A) (n= 11) and SakSTAR(K74A,E75A,R77A) (n= 6) combined with previous experience in 14 patients with SakSTAR (n= 7) and SakSTAR(K35A,E38A,K74A,E75A,R77A) (n= 7) (31) and in 24 patients with SakSTAR (32), and with subsequent non-randomized experience in patients with SakSTAR (n= 30) with SakSTAR(K74A) (n= 12) and with SakSTAR(K74A,E75A,R77A) (n= 7) (data not shown), allows an initial estimation of the prevalence of immunization by intra-arterial treatment with SakSTAR or variants with an altered K74,E75,R77 epitope [SakSTAR(K74A), SakSTAR(K74A,E75A,R77A) and SakSTAR(K35A,E38A,K74A,E75A, R77A)]. Neutralizing activity data after 2 to 4 weeks, available in 70 patients with peripheral arterial occlusion given intra-arterial SakSTAR, revealed that 56 patients (80 percent) had levels > 5 µg compound neutralized per mL plasma. Of the patients given SakSTAR(K74A), SakSTAR(K74A,E75A,R77A) or SakSTAR(K74A,E75A,K74A,E75A,R77A), 27 of the 43 (63 percent) had neutralizing activity levels of > 5 µg compound per mL plasma. This difference is statistically significant (p= 0.05 by Fisher's exact test) indicating that the K74,E75,R77 epitope is a major determinant of antibody induction. However, the residual

purification kit from Qiagen (Hilden, Germany) or the BIO 101 RPM kit (Vista, CA), as recommended. Transformation-competent *E. coli* cells were prepared by the well-known calcium phosphate procedure. Nucleotide sequence determination was performed on double strand plasmid DNA with the dideoxy chain termination method, using the T7 sequencing kit (Pharmacia, Uppsala, Sweden). Polymerase chain reactions (PCR) were performed using Taq polymerase from Boehringer Mannheim (Mannheim, Germany) or Vent polymerase (New England Biolabs, Leusden, The Netherlands). The recombinant DNA methods required to construct the variants described in this example are well established (22,27).

Construction of expression plasmids

The variants SakSTAR(Y17A,F18A), SakSTAR(F104A), SakSTAR(F111A), SakSTAR(Y9A), SakSTAR(Y91A), SakSTAR(Y92A), SakSTAR(I87A), SakSTAR(I106A) and SakSTAR(I120A) were constructed with the Chameleon Double-Stranded Site-Directed Mutagenesis kit from Stratagene (La Jolla, USA), using the *pMEX.SakSTAR* vector as template, and following instructions of the supplier. The mutagenic oligonucleotides (not shown) were used in combination with the selection-primer LY34 5' CAAAACAGCCGAGCTTCATTCATTCAGC, which destroys the unique *Hin*dIII site located 3' to the staphylokinase encoding gene in *pMEX.SakSTAR* and allows to counter-select the non-mutant progeny by HindIII digestion. The deletion of the *Hin*dIII site was in most cases correlated with the presence of the desired mutation introduced by the mutagenic oligonucleotide. The variant SakSTAR(I133A), was constructed by performing a polymerase chain reaction on the *pMEX.SakSTAR* plasmid using the primer 818A located at the 5' end of the *sak*STAR gene (5' CAGGAAACAGAATTCAGGAG) and the mutagenic primer LY58 (5' TTCAGCATGCTGCAGTTATTTCTTTTCTGCAACAACCTTGG). The amplified product (30 cycles: 30 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C) was purified, digested with EcoRI and PstI, and ligated into the corresponding sites of *pMEXSakSTAR*.

The variants SakSTAR(I128A), SakSTAR(L127A) and SakSTAR(N126V) were constructed by performing a polymerase chain reaction using the primer 818A located at the 5' end of the *sakSTAR* gene and mutagenic primers (not shown). The amplified product (30 cycles: 1 sec at

and at 30°C. After about 16 hours incubation, IPTG (200 µmol/L) was added to the culture to induce expression from the *tac* promoter. After 3 hours induction, the cells were pelleted by centrifugation at 4,000 rpm for 20 min, resuspended in 1/10 volume of 0.01 mol/L phosphate buffer pH 6-6.5 and disrupted by sonication at 0°C. The suspension was centrifuged for 20 min at 20,000 rpm and the supernatant was stored at 4°C or at -20°C until purification. The material was purified essentially as described above (Example 2): cleared cell lysates containing the SakSTAR variants were subjected to chromatography on a 1.6 x 5 cm column of SP-Sephadex, followed by chromatography on a 1.6 x 8 cm column of phenyl-Sepharose. The SakSTAR containing fractions, localized by SDS-gel electrophoresis, were pooled for further analysis.

Physicochemical and biochemical analysis

Protein concentrations were determined according to Bradford (29). SDS-PAGE was performed with the Phast System™ (Pharmacia, Uppsala, Sweden) using 10-15% gradient gels and Coomassie Brillant blue staining, and the specific activities of SakSTAR solutions were determined with a chromogenic substrate assay carried out in microtiter plates (as described in example 2). The specific activity of the different SakSTAR variants are summarized in Table 3.

Reactivity of SakSTAR variants with a panel of murine monoclonal antibodies

The methodology used to determine the reactivity of the SakSTAR variants with a panel of murine monoclonal antibodies was described in example 1 above. The results are summarized in Table 3 (the layout of this Table corresponds to the layout of Table 1, as described in example 1). Apparent association constants at least 10-fold lower than those of wild-type staphylokinase were considered as significant and are indicated in bold type in the table.

In order to obtain a comprehensive inventory of the properties of Ala-substitution variants of the SakSTAR molecule, 67 plasmids encoding variants with substitution of a single or two adjacent amino acids with Ala were constructed, expressed and purified. Together with the 35

EXAMPLE 5

Construction, epitope mapping with murine mon clonal antibodies and absorption with pooled plasma of immunized patients, of staphylokinase variants with substitution of S34, G36 and/or H43

The natural variant Sak42D differs from SakSTAR in three amino acids and corresponds to SakSTAR(S34G,G36R,H43R). Sak42D is characterized by reduced reactivity with some murine antibodies of epitope clusters II and III and a slightly reduced absorption of antibodies from plasma of patients treated with SakSTAR (Table 4). Mutagenesis of these residues in SakSTAR revealed that the reduced reactivity with epitope cluster III and with immunized patient plasma could be ascribed to the G36R substitution, the H43R substitution mediated the reduced reactivity with epitope cluster II but had no effect on the reactivity with immunized patient plasma, whereas the S34A substitution had no effect. The G36R substitution could be combined with the K74R but not with the K74A substitution, without significant reduction of the specific activity (Table 4).

EXAMPLE 6

Construction, epitope mapping with murine monoclonal antibodies and absorption with pooled plasma of immunized patients, of staphylokinase variants with substitution of K35, E65, Y73, K74, E80+D82, N95, K130, V132 and/or K135

Based on the results of the alanine-substitution analysis in example 4, K35, N95 and K135 were selected for further analysis because SakSTAR(K35A), SakSTAR(N95A) and SakSTAR(K135A) had a two-fold increased specific activity, Y73 and K74 because SakSTAR(Y73A) and SakSTAR(K74A) had a markedly reduced reactivity with antibodies from epitope cluster I and diminished absorption of antibodies from plasma of patients immunized by treatment with SakSTAR, and K35, E80+D82, K130 and V132 because SakSTAR(K35A), SakSTAR(E80A,D82A), SakSTAR(K130A) and SakSTAR(V132A) had a reduced reactivity with antibodies from epitope cluster III.

SakSTAR(K130T,K135R) template reduced the absorption of antibodies from Pool 10 and Pool 40 to around 50 and 60 percent respectively, without markedly reducing the specific activity. Addition substitution of selected amino acids in the SakSTAR(E65Q,K74Q,K130T,K135R) template did not further reduce the antibody absorption from Pool 10 or Pool 40. Surprisingly, the substitution of K136 with A and the addition of K in position 137 resulted in a marked increase in specific activity, as measured in the chromogenic substrate assay.

Combination of the SakSTAR(E80A,D82A) and SakSTAR(K130T,K135R) templates, did not affect the specific activity and had a reduced reactivity with epitope cluster III antibodies (Table 7). Therefore the SakSTAR(E80A,D82A,K130T,K135R) template was selected for further mutagenesis. Addition of K74R and even more of K74Q drastically reduced the reactivity with immunized patient plasma. Finally, addition of E65D or of K35A or E65S to the SakSTAR(K74R,E80A,D82A,K130T,K135R) or SakSTAR(K74Q,E80A,D82A,K130T, K135R) templates yielded variants with intact specific activity which only bound ≤45 of the antibodies of pooled immunized patient plasma and less than 15 percent of the subpool reacting for more than 50 percent with the K74,E75,R77 epitope.

EXAMPLE 8

Characterization of selected variants of staphylokinase with intact specific activity and less than 50% adsorption of pooled SakSTAR specific human antibodies elicited in patients by treatment with wild-type SakSTAR.

Twenty three of the variants constructed and characterized in the above examples combined the properties of a residual specific activity of ≥ 100 kHU/mg and ≤ 50 percent absorption with the pool of antisera obtained from 10 patients treated with wild-type SakSTAR. The results are summarized in Table 8. Results obtained with Subpool B and Subpool C and with the pool of 40 patients treated with wild-type SakSTAR are included. SakSTAR(K74Q,E80A,D82A,K130T,K135R), SakSTAR(E65D,K74R,E80A,D82A,K130T, K135R), SakSTAR(K35A,E65D,K74Q,E80A,D82A,K130T,K135R) and SakSTAR(E65Q,

Pharmacokinetic properties of SakSTAR variants following bolus injection in hamsters

The pharmacokinetic parameters of the disposition of SakSTAR variants from blood were evaluated in groups of 4 hamsters following intravenous bolus injection of 100 µg/kg SakSTAR variant. SakSTAR-related antigen was assayed using the ELISA described elsewhere. The ELISA was calibrated against each of the SakSTAR variants to be quantitated. Pharmacokinetic parameters included: initial half-life (in min), $t1/2\alpha = \ln 2/\alpha$; terminal half-life (in min), $t1/2\beta = \ln 2/\beta$; volume of the central (plasma) compartment (in mL), V_C= dose/(A+B); area under the curve (in $\mu g.min.mL^{-1}$), AUC= $A/\alpha + B/\beta$; and plasma clearance (in $mL.min^{-1}$), Clp= dose/AUC (33).

The disposition rate of staphylokinase-related antigen from blood following bolus injection of 100 µg/kg of the selected SakSTAR variants in groups of 4 hamsters could adequately be described by a sum of two exponential terms by graphical curve peeling (results not shown), from which the pharmacokinetic parameters summarized in Table 10 were derived. The pharmacokinetic parameters of the mutants were not markedly different from those of wild type SakSTAR. Initial plasma half-lives ($t1/2(\alpha)$) ranged between 2.0 and 3.2 min and plasma clearances (Clp) between 1.6 and 4.1 mL/min.

EXAMPLE 9

Comparative thrombolytic efficacy and immunogenicity of SakSTAR(K74Q, E80A,D82A,K130T,K135R) and SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) versus SakSTAR in patients with peripheral arterial occlusion

Purification for use in vivo

Eighteen liter cultures (in 2 L batches) of the variants SakSTAR(K74Q,E80A,D82A,K130T, K135R) and SakSTAR(E65D,K74R,E80A,D82A, K130T,K135R) were grown for 20 hours in terrific broth medium (28), supplemented with 100 µg/mL ampicillin and induced with IPTG during the last 3 hours. The cells were pelleted, resuspended in 1/10 volume of 0.01 mol/L phosphate buffer, pH 6.0, disrupted by sonication and cleared by centrifugation. The

in the proximal end of the occlusive thrombus as a bolus of 2 mg followed by an infusion of 1 mg/hr (reduced overnight in some patients to 0.5 mg/hr) in groups of 15, 6 and 6 patients respectively with angiographically documented occlusion of a peripheral artery or bypass graft of less than 30 days duration. Patients were studied after giving informed consent, and the protocol was approved by the Human Studies Committee of the University of Leuven. Inclusion and exclusion criteria, conjunctive antithrombotic treatment (including continuous intravenous heparin) and the study protocol were essentially as previously described (22).

Relevant baseline characteristics of the individual patients and results of treatment and outcome are shown in Table 11. Intra-arterial infusion, at a dose of 3.5 to 27 mg and a duration of 2 to 44 hrs, induced complete recanalization in 22 patients and partial recanalization in 5. Complementary endovascular procedures (mainly PTA) were performed in 13 patients and complementary reconstructive vascular surgery following thrombolysis in 5. One patient underwent major amputation. Bleeding complications were usually absent or limited to mild to moderate hematoma formation at the angiographic puncture sites (data not shown). One patient, given wild-type SakSTAR suffered a non-fatal intracranial bleeding, one (BUE) a retroperitoneal hematoma and two (MAN and STRO) a gastro-intestinal bleeding.

Circulating fibrinogen, plasminogen and $\alpha 2$-antiplasmin levels remained unchanged during infusion of the SakSTAR moieties (data not shown), reflecting absolute fibrin specificity of these agents at the dosages used (data not shown). Significant in vivo fibrin digestion occurred as evidenced by elevation of fibrin fragment D-dimer levels. Intra-arterial heparin therapy prolonged aPTT levels to a variable extent (data not shown).

Antibody induction

Staphylokinase-neutralizing activity in plasma and antigen-specific IgG antibodies were quantitated essentialy as described above and elsewhere (22).

Antibody-related SakSTAR-, SakSTAR(K74Q,E80A,D82A,K130T,K135R)- and SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R)-neutralizing activity and anti-SakSTAR, anti-SakSTAR(K74Q,E80A,D82A,K130T,K135R) and anti-

EXAMPLE 10

Constructi n, purification and characterization of cysteine-substitution mutants of staphylokinase

Site-directed mutagenesis was applied to substitute exposed amino acids with single cysteine residues in order to construct i) homodimeric forms of staphylokinase, upon formation of an intermolecular disulfide bridge, and ii) polyethylene glycol-conjugated molecules (PEG-derivatives). The aim for this study is twofold: first, the clearance can be reduced by increasing the size of the injected molecule (via dimerization or conjugation with large molecule such as PEG) and second, PEG-derivatives have also been shown to induce a reduced immunoreactivity in animal models (for review, see ref. 34). In both cases, a prolonged half-life *in vivo* could help to reduce the pharmacological dosis of straphylokinase in patients. This reduction could be accompanied with a reduced immunogenic reaction against the thrombolytic agent.

In this example, the construction and characterization of two SakSTAR variants in which one single amino acid was substituted with cysteine is described. The mutants described under this example are listed in Table 13. These variants were expressed in *E. coli*, purified and characterized to some extent in terms of specific activity, fibrinolytic properties in human plasma *in vitro* and pharmacokinetic properties following bolus injection in hamsters.

Reagents and Methods

The source of all reagents used in the present study has previously been reported (22), or is specified below. The template vector for mutagenesis, *pMEX602sakB* (i.e. *pMEX.SakSTAR*), has been described elsewhere (23). Restriction and modification enzymes were purchased from New England Biolabs (Leusden, The Netherlands), Boehringer Mannheim (Mannheim, Germany) or Pharmacia (Uppsala, Sweden). The enzymatic reactions were performed according to the supplier recommendation. The mutagenic oligonucleotides and primers were obtained from Eurogentec (Seraing, Belgium). Plasmid DNA was isolated using a purification kit from Qiagen (Hilden, Germany), as recommended. Transformation-competent *E. coli* cells were prepared by the well-known calcium phosphate procedure.

induce expression from the *tac* promoter. After 3 hours induction, the cells were pelleted by centrifugation at 4,000 rpm for 20 min, resuspended in 1/10 volume of 0.01 mol/L phosphate buffer pH 6-6.5 and disrupted by sonication at 0°C. The suspension was centrifuged for 20 min at 20,000 rpm and the supernatant was stored at 4°C or at -20°C until purification. The material was purified essentially as described above (Example 2): cleared cell lysates containing the SakSTAR variants were subjected to chromatography on a 1.6 x 5 cm column of SP-Sephadex, followed by chromatography on a 1.6 x 8 cm column of phenyl-Sepharose. The SakSTAR containing fractions, localized by SDS-gel electrophoresis, were pooled for further analysis.

Biochemical analysis

Protein concentrations were determined according to Bradford (29). SDS-PAGE was performed with the Phast System™ (Pharmacia, Uppsala, Sweden) using 10-15% gradient gels and Coomassie Brillant blue staining, and the specific activities of SakSTAR solutions were determined with a chromogenic substrate assay carried out in microtiter plates (as described in example 2). The specific activity of the different SakSTAR variants are summarized in Table 13.

Mutant SakSTAR(K102C) was essentially monomeric as visualized by SDS-PAGE and Coomassie Brillant blue staining. Its specific activity was comparable to that of wild-type staphylokinase. In contrast, SakSTAR(K109C) showed a propensity to form dimers (> 60%). This resulted in a markedly increased specific activity in the plasminogen-coupled chromogenic substrate assay (see Table 13). Upon reduction with dithiothreitol (DTT) (20-fold molar excess during 1.5 hour at 37°C) and alkylation with iodoacetamide (100-fold molar excess during 1 hour at 37°C), the K109C dimer is converted into a stable monomer and its resulting specific activity is within the expected range towards wild-type staphylokinase (Table 13). This results confirms that formation of homodimers is the unique determinant for this large increase in specific activity. Dimeric SakSTAR(K109C) was separated from monomeric SakSTAR(K109C) by chromatography on Source S (Pharmacia) (5 x 50mm). Loading buffer was 10 mM phosphate, pH 6.0 and dimeric SakSTAR(K109C)

comparable to that of SakSTAR, for monomeric SakSTAR(K109C) and SakSTAR(K102C) (Table 13). However, it was observed that the C_{50} for clot lysis by dimeric SakSTAR(K109C) was only 0.12 µg/ml, which is approximately three-fold lower than for wild-type staphylokinase. In contrast, a C_{50} of 0.60 µg/ml was measured for SakSTAR(K102C-PEG), which is only two-fold higher than for wild-type staphylokinase. Thus, dimerization of SakSTAR via disulfide bridges or increasing the size of the molecule via PEG-derivatization does not preclude the fibrinolytic activity of staphylokinase. While a PEG-molecule appears to reduce the diffusion and therefore fibrinolytic potency of the derivatized staphylokinase within a fibrin clot, dimerization of staphylokinase results in a synergistic fibrinolytic effect on human fibrin clots.

Pharmacokinetic properties of dimeric SakSTAR(K109C) and SakSTAR(K102C-PEG) following bolus injection in hamsters

The pharmacokinetic parameters of the disposition of dimeric SakSTAR(K109C) and SakSTAR(K102C-PEG) from blood were evaluated in groups of 4 hamsters following intravenous bolus injection of 100 µg/kg SakSTAR variant. SakSTAR-related antigen was assayed using the ELISA described elsewhere. The ELISA was calibrated against each of the SakSTAR variants to be quantitated. Pharmacokinetic parameters included: initial half-life (in min), $t1/2\alpha = \ln 2/\alpha$; terminal half-life (in min), $t1/2\beta = \ln 2/\beta$; volume of the central (plasma) compartment (in mL), V_C= dose/(A+B); area under the curve (in µg.min.mL^{-1}), AUC= $A/\alpha + B/\beta$; and plasma clearance (in mL.min^{-1}), Clp= dose/AUC (32).

The disposition rate of staphylokinase-related antigen from blood following bolus injection of 100 µg/kg of the selected SakSTAR variants in groups of 4 hamsters could adequately be described by a sum of two exponential terms by graphical curve peeling (results not shown), from which the pharmacokinetic parameters $t1/2\alpha$ and Clp, summarized in Table 13 were derived. The pharmacokinetic parameters of dimeric SakSTAR(K109C) and SakSTAR(K102C-PEG) were markedly different from those of wild type SakSTAR. Initial plasma half-lives ($t1/2(\alpha)$) were 3.6 and 3.0 min and plasma clearances (Clp) were 0.52 and 0.32 mL/min, for dimeric SakSTAR(K109C) and SakSTAR(K102C-PEG), respectively.

CONCLUSION

In summary, the present experience illustrates that staphylokinase variants with markedly *reduced* antibody induction but *intact* thrombolytic potency can be generated. To our knowledge, this observation constitutes the first case in which a heterologous protein, with the use of protein engineering techniques, is rendered significantly less immunogenic in man without reducing its biological activity.

The present invention was inititated by the observation that certain "clustered charge-to-alanine" substitution variants of recombinant staphylokinase (SakSTAR variant (9)) had a reduced reactivity with antibodies induced by treatment with wild type SakSTAR (3,4) and induced less antibodies than wild type SakSTAR in patients with peripheral arterial occlusion (22,32,35). In an effort to optimize the specific activity versus antigenicity ratio, a comprehensive mutagenesis study, comprising the construction and expression of over 250 plasmids encoding SakSTAR variants, and the purification of the translation products was undertaken. The SakSTAR variants were characterized in terms of specific activity, affinity towards a panel of murine monoclonal antibodies and absorption of SakSTAR specific antibodies from pooled plasma of 10 patients treated with wild type SakSTAR and of two subpools of 3 patients each which reacted strongly (subpool B) or poorly (subpool C) with the immunodominant epitope K74,E75,R77. In a later phase, an additional pool of 40 patients treated with wild-type SakSTAR was also used for absorption studies. The values obtained with both pools were in good agreement. Linear regression analysis yielded

(Pool 40)= 0.84 x (Pool 10) + 14, with r= 0.94 and n= 61.

Residues for site-directed mutagenesis were selected in three ways: 1) a comprehensive analysis of variants with 1 or 2 adjacent amino acids substituted with Ala; 2) analysis of the differential reactivity of the two natural variants SakSTAR and Sak42D (which corresponds to SakSTAR(S34G,G36R,H43R) and 3) surface exposure of the residues as derived from the three dimensional structure. From these analyses, SakSTAR(K35A), SakSTAR(N95A) and SakSTAR(S103A) emerged with specific activities ≥200 kU/mg. SakSTAR(W66A), SakSTAR(Y73A) and SakSTAR(E75A) with reduced reactivity with ≥3 of the 5 antibodies

patient plasma. Finally, addition of E65D or E65Q to the SakSTAR(K74R,E80A,D82A, K130T,K135R) template yielded variants with intact specific activity which only bound 1/3 of the antibodies of pooled immunized patient plasma, only about 10 to 30 percent of the antibodies from plasma of patients with a high concentration of antibodies directed towards the immunodominant K74,E75,R77 (Subpool B) and only about 60 percent of the antibodies from plasma of patients with a very low concentration of antibodies directed against this immunodominant epitope (Subpool C). Based on this analysis, SakSTAR(K74Q,E80A, D82A,K130T,K135R), and SakSTAR(E65D,K74R,E80A, D82A,K130T,K135R) were selected for further analysis.

The fibrinolytic potency and the fibrin-selectivity of these selected mutants in a plasma milieu was indistinguishable from that of wild type SakSTAR. The temperature stability of the mutants was still acceptable with no significant loss of activity upon incubation at 37°C for 3 days, although at 56° and 70°C, they were more rapidly inactivated than wild-type SakSTAR. The pharmacokinetics of the SakSTAR variants following intravenous bolus injection in hamsters did not reveal major differences with wild type SakSTAR except for a possibly somewhat higher plasma clearance.

In conclusion, the two variants of SakSTAR which emerged from the present site directed mutagenesis program are characterized by an intact or slightly increased specific activity, maintained thrombolytic potency and fibrin-selectivity in a human plasma milieu, acceptable although slightly reduced temperature stability and a markedly reduced reactivity with anti-SakSTAR antibodies in pooled immunized patient plasma. In view of the previously found correlation between reduced antigenicity and reduced immunogenicity of certain "charged-cluster-to-alanine" variants investigation of immunogenicity associated with their use for thrombolytic therapy in man appeared warranted.

Highly purified, sterilized preparations of SakSTAR(K74Q,E80A,D82A,K130T,K135R) and SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) were produced and found to contain low endotoxin levels, and to be devoid of acute toxicity in mice following intravenous bolus injection at a dose of 3 mg/kg.

REFERENCES

1. Lack CH: Staphylokinase: an activator of plasma protease. Nature 161: 559, 1948.
2. Lewis JH, Ferguson JH: A proteolytic enzyme system of the blood. III. Activation of dog serum profibrinolysin by staphylokinase. Am J Physiol 166: 594, 1951.
3. US5336495 (issued 94.08.09).
4. Vanderschueren S, Barrios L, Kerdsinchai P, Van den Heuvel P, Hermans L, Vrolix M, De Man F, Benit E, Muyldermans L, Collen D, Van de Werf F: A randomized trial of recombinant staphylokinase versus alteplase for coronary artery patency in acute myocardial infarction. Circulation 92: 2044-2049, 1995.
5. Sako T, Sawaki S, Sakurai T, Ito S, Yoshizawa Y, Kondo I: Cloning and expression of the staphylokinase gene of Staphylococcus aureus in Escherichia coli. Molec Gen Genet 190: 271-277, 1983.
6. Behnke D, Gerlach D: Cloning and expression in Escherichia coli, Bacillus subtilis, and Streptococcus sanguis of a gene for staphylokinase - a bacterial plasminogen activator. Molec Gen Genet 210: 528-534, 1987.
7. Collen D, Silence K, Demarsin E, De Mol M, Lijnen HR: Isolation and characterization of natural and recombinant staphylokinase. Fibrinolysis 6: 203-213, 1992.
8. Sako T, Tsuchida N: Nucleotide sequence of the staphylokinase gene from Staphylococcus aureus. Nucleic Acids Res 11: 7679-7693, 1983.
9. Collen D, Zhao ZA, Holvoet P, Marynen P: Primary structure and gene structure of staphylokinase. Fibrinolysis 6: 226-231, 1992.
10. Sakai M, Watanuki M, Matsuo O: Mechanism of fibrin-specific fibrinolysis by staphylokinase: participation of α_2-plasmin inhibitor. Biochem Biophys Res Comm 162: 830-837, 1989.
11. Matsuo O, Okada K, Fukao H, Tomioka Y, Ueshima S, Watanuki M, Sakai M: Thrombolytic properties of staphylokinase. Blood 76: 925-929, 1990.
12. Lijnen HR, Van Hoef B, De Cock F, Okada K, Ueshima S, Matsuo O, Collen D: On the mechanism of fibrin-specific plasminogen activation by staphylokinase. J Biol Chem 266: 11826-11832, 1991.

of recombinant staphylokinase for thrombolytic therapy. Bio/technology 12: 185-189, 1994.

24. Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77: 61-68, 1989.
25. BIAcore system manual, 5-2, Pharmacia Biosensor AB, Uppsala, Sweden.
26. Karlsson R, Michaelsson A, Mattsson L: Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system. J Immunol Methods 145: 229-240, 1991.
27. Sambrook J, Fritsch EF, Maniatis T: Molecular cloning: a laboratory mannual. 2nd Ed. Cold Spring Harbor, NY. Cold Spring Harbor Laboratory Press, 1989.
28. Tartof KD, Hobbs CA: Improved media for growing plasmid and cosmid clones. Bethesda Res Lab Focus 9: 12, 1987
29. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248, 1976.
30. Deutsch DG, Mertz ET: Plasminogen: purification from human plasma by affinity chromatography. Science 170: 1095-1096, 1970.
31. Collen D, Moreau H, Stockx L, Vanderschueren S. Recombinant staphylokinase variant with altered immunoreactivity. II. Thrombolytic properties and antibody induction. Circulation 94: 207-216, 1996.
32. Vanderschueren S, Stockx L, Wilms G, Lacroix H, Verhaeghe R, Vermylen J, Collen D: Thrombolytic therapy of peripheral arterial occlusion with recombinant staphylokinase. Circulation 92: 2050-2057, 1995.
33. Gibaldi M, Perrier D. Pharmacokinetics, Marcel Dekker, New York, N.Y., 1983, 45-111.
34. Inada Y, Furukawa M, Sasaki H, Kodera Y, Hiroto M, Nishimura H, Matsushima A. Biomedical and biotechnological applications of PEG- and PM-modified proteins, TIBTECH 13: 86-91, 1995.

Table 1: Alanine-to-wild-type" reversal variants of "charged-cluster-to-alanine" mutants of SakSTAR: Association constants (K_A x 10^7 mol/L^{-1}) for the binding to insolubilized murine monoclonal antibodies (Mabs), and absorption (percent) of antibodies of immunized patient plasma

riant	Exp. (mg/L)	Spec. Act. (kU/mg)	murine MAbs: Epitope I					Epitope II					Epitope III					SakSTAR patient plasma		
			17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool	Subpool B	Subpool C
:STAR		130	22	13	2.9	7.8	11	38	7.4	19	7.7	2.4	4.0	14	5.4	2.9	0.6	95	95	95
SakSTAR(K35A,E38A)		97	15	22	4.2	11	7.9	110	10	15	12	2.2	**<0.1**	**<0.1**	**<0.1**	1.0	1.0	93	91	94
SakSTAR(K74A,E75A,R77A)		110	11	**<.01**	**<0.1**	**<0.1**	**<0.1**	150	17	28	14	3.3	2.4	11	4.0	2.1	0.9	**55**	**43**	95
SakSTAR(K35A,E38A,K74A,E75A,R77A)		50	11	**<0.1**	**<0.1**	**<0.1**	**<0.1**	110	36	26	15	2.0	**<0.1**	**<0.1**	**<0.1**	1.5	1.2	**52**	**41**	92
SakSTAR(E38A,K74A,E75A,R77A)		43	11	**<0.1**	**<0.2**	**<0.1**	**<0.1**	140	39	26	15	2.1	**<0.1**	3.2	3.7	1.6	1.1	**50**	**44**	95
SakSTAR(K35A, K74A,E75A,R77A)		56	9.2	**<0.1**	**0.15**	**<0.1**	**<0.1**	52	14	29	8.8	2.3	**<0.1**	1.8	**<0.1**	1.8	0.8	**46**	**43**	95
SakSTAR(K35A,E38A,E75A,R77A)		44	11	**0.3**	**0.1**	**0.2**	**<0.1**	75	9.8	12	7.3	1.6	**<0.1**	**<0.1**	**<0.1**	0.53	0.64	92	87	94
SakSTAR(K35A,E38A,K74A,R77A)		41	8.8	2.9	**<0.1**	2.0	**0.33**	110	29	31	10	2.0	**<0.1**	**<0.1**	**<0.1**	0.63	0.74	**56**	**50**	93
SakSTAR(K35A,E38A,K74A,E75A)		19	13	**<0.1**	**0.1**	**<0.1**	**<0.1**	180	41	37	15	1.6	**<0.1**	**<0.1**	**<0.1**	1.2	0.45	**48**	**41**	92
SakSTAR(E38A,E75A,R77A)		88	11	**0.6**	**0.15**	**0.4**	**0.3**	79	12	15	10	2.0	**<0.1**	2.6	4.7	1.1	0.81	95	88	95
SakSTAR(E38A,E75A)		66	16	**0.3**	**<0.1**	**<0.1**	**0.9**	56	11	13	8.9	2.0	**<0.1**	20	4.8	1.3	1.6	91	90	95
SakSTAR(K35A,E75A,R77A)		68	9.2	**<0.1**	**<0.1**	**<0.1**	**<0.1**	60	7.0	13	11	3.3	**<0.1**	1.5	**<0.1**	0.8	1.1	88	89	95
SakSTAR(K35A,E75A)		150	17	**0.12**	**<0.1**	**0.16**	**0.14**	40	7.2	13	9.2	4.2	**<0.1**	1.8	**<0.1**	1.4	1.5	94	93	95
SakSTAR(K74A)		100	12	7.6	**0.17**	4.4	2.1	55	15	33	14	3.6	2.9	14	4.9	3.4	1.2	**59**	**45**	95
SakSTAR(E75A)		140	13	**1.2**	**<0.1**	**<0.1**	**<0.1**	46	8.5	14	12	3.4	4.5	18	5.0	1.2	2.1	95	93	95
SakSTAR(K74A,E75A,R77A,E80A,D82A)		50	14	**<0.1**	**<0.1**	**<0.1**	**<0.1**	180	19	33	19	3.7	**<0.1**	**<0.1**	**<0.1**	**<0.1**	1.2	**49**	**29**	89
SakSTAR(E80A,D82A)		130	7.3	12	2.1	6.5	5.9	79	6.1	8.4	7.8	1.9	**<0.1**	**<0.1**	**<0.1**	**<0.1**	0.44	89	83	92
SakSTAR(E80A)		160	13	13	3.3	7.9	10	35	7.4	17	8.6	2.1	**<0.1**	16	3.6	**<0.1**	1.7	94	93	95
SakSTAR(D82A)		160	17	12	4.8	7.3	11	31	7.8	17	12	2.7	**<0.1**	**0.18**	**<0.1**	**<0.1**	2.3	95	93	95
SakSTAR(E75A,D82A)		170	20	15	3.1	6.6	7.2	69	8.1	15	14	4.9	**0.17**	**0.7**	**0.5**	**0.1**	1.4	95	95	95

Apparent association constants ≥ 10-fold lower than those of wild-type SakSTAR are represented in bold type; Spec. Act. ≥ 100.000 HU/mg represented in bold type; ≤60% absorption represented in bold type.

Table 3: Alanine-substitution variants of SakSTAR: Association constants (K_A x $10^7 mol/L^{-1}$) for binding to insolubilized murine monoclonal antibodies (Mab) and absorption (percent) of antibodies of immunized patient plasma

			murine MAbs														SakSTAR patient plasma		
			Epitope cluster I					Epitope cluster II					Epitope cluster III						
	Exp. (mg/L)	Spec. Act. (kU/mg)	17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool	Subpool B	Subpool C
SakSTAR		120	9.3	13	2.9	7.8	11	38	7.4	19	7.7	2.4	4.0	14	5.4	2.9	0.6	95	95	95
			[6.4	17	7.4	19	35	18	25	>18	>14	1.1	0.6	11	16	6.3	2.0]			
SakSTAR(S34G,G36R,H43R)		120	10	14	3.3	7.5	11	<0.1	<0.1	<0.1	20	2.7	<0.1	<0.1	<0.1	0.15	1.7	87	76	75
SakSTAR(F4A)	LE																			
SakSTAR(D5A,K6A)		150	[1.3	21	12	9.2	9.7	12	23	17	10	0.4	1.3	3.9	0.8	4.5	3.8]	95	95	95
SakSTAR(K8A,K10A)		24	[1.8	16	5.1	29	15	22	16	26	18	1.0	0.93	11	1.1	18	0.75]	95	95	95
SakSTAR(Y9A)	24	78	22	49	6.6	2.3	16	44	8.4	20	14	2.6	2.4	16	9.6	1.1	0.5	96	95	95
SakSTAR(K11A,D13A,D14A)	LE																			
SakSTAR(D13A)	6	46	2.4	6.1	2.0	3.7	3.4	11	1.9	4.5	4.4	8.7	1.5	2.4	1.1	3.6	<0.1	95	94	95
SakSTAR(D14A)	14	30	5.1	13	4.0	6.6	3.1	38	7.7	12	15	2.2	2.7	6.0	3.2	5.6	<0.1	95	94	95
SakSTAR(S16A)	41	160	8.1	16	4.5	8.4	9.0	15	6.1	13	21	3.6	4.0	8.0	4.3	2.5	0.5	95	95	95
SakSTAR(Y17A,F18A)	27	30	13	22	3.3	10	9.5	21	4.6	6.7	12	2.5	1.2	18	6.5	3.4	<0.1	95	95	95
SakSTAR(E19A,P20A)	36	9	11	19	3.3	9.2	12	15	6.1	11	16	1.0	1.1	15	5.1	3.3	<0.1	93	93	95
SakSTAR(T21A)	54	170	4.8	15	2.4	8.7	9.6	32	11	24	18	1.3	1.8	9.6	2.9	5.6	0.6	95	95	95
SakSTAR(P23A)	41	67	14	31	4.4	14	22	41	5.3	37	13	10	4.0	11	7.6	3.1	1.9	91	95	95
SakSTAR(Y24A)	10	40	17	33	4.3	13	11	33	4.3	7.0	12	4.0	0.4	14	6.8	4.8	<0.1	95	95	95
SakSTAR(L25A)	LE																			
SakSTAR(M26A)	LE																			
SakSTAR(V27A)	62	50	3.3	15	1.6	7.8	7.4	12	2.9	3.7	33	2.0	1.3	5.9	2.8	4.2	1.2	95	95	95
SakSTAR(N28A)	90	<5	5.8	29	2.1	7.0	5.5	27	10	20	2.5	2.1	2.1	5.6	2.1	2.1	0.7	95	95	95
SakSTAR(N28A,V29A)	32	45	18	30	2.5	20	18	20	14	20	24	2.7	3.3	20	1.1	5.0	2.0	93	95	95
SakSTAR(T30A)	52	140	7.4	13	2.1	7.0	6.1	7.6	3.4	5.1	13	3.3	5.6	12	3.4	5.4	0.8	94	95	95
SakSTAR(V32A)	78	45	10	9.6	2.4	6.2	7.8	56	17	12	14	1.4	<0.1	<0.1	<0.1	<0.1	2.2	90	93	95
SakSTAR(D33A,K35A)		130	[2.1	19	14	14	19	15	24	32	10	5.3	1.4	5.1	3.8	5.1	3.0]	95	95	95
SakSTAR(S34A)	29	110	17	24	4.6	9.5	11	28	11	22	15	2.9	3.1	8.8	3.8	2.0	0.2	95	95	93

Table 3 - cont'd: Alanine-substitution variants of SakSTAR: Association constants (K_A x $10^7 mol/L^{-1}$) for binding to insolubilized murine monoclonal antibodies (Mab) and absorption (percent) of antibodies of immunized patient plasma

nt	Exp. (mg/L)	Spec. Act. (kU/mg)	murine MAbs: Epitope cluster I 17G11	26A2	30A2	2B12	3G10	Epitope cluster II 18F12	14H5	28H4	32B2	7F10	Epitope cluster III 7H11	25E1	40C8	24C4	1A10	SakSTAR patient plasma: Pool	Subpool B	Subpool C
TAR(Y73A)	20	<5	9.5	<0.1	<0.1	<0.1	4.8	33	7.3	11	8.9	2.0	0.5	9.0	2.5	1.4	0.8	63	44	93
TAR(Y73A,K74A)	24	<5	18	<0.1	<0.1	<0.1	<0.1	19	6.7	23	9.9	3.2	2.7	13	4.0	1.6	1.1	47	28	87
SakSTAR(K74A)	80	69	4.4	2.7	0.2	2.2	1.1	17	5.2	14	7.6	2.2	2.0	6.8	3.3	1.8	0.9	64	58	95
SakSTAR(K74A,E75A,R77A)		68	9.2	<0.1	<0.1	<0.1	<0.1	60	7.0	13	11	3.3	<0.1	1.5	<0.1	0.8	1.1	88	89	95
SakSTAR(K74A,R77A)	34	41	3.5	1.8	0.2	1.5	0.4	20	2.4	10	2.1	1.8	1.7	2.3	2.2	1.2	0.7	74	60	95
SakSTAR(E75A)		140	13	1.2	<0.1	<0.1	<0.1	46	8.5	14	12	3.4	4.5	18	5.0	1.2	2.1	95	93	95
SakSTAR(F76A)	9	90	20	9.6	1.0	2.7	3.9	13	6.2	20	15	1.7	0.3	5.9	2.1	1.2	1.0	94	92	95
SakSTAR(V78A,V79A)	23	68	12	23	4.0	10	17	21	18	34	28	2.3	1.6	4.7	<0.1	0.5	1.7	93	93	95
SakSTAR(E80A)		160	13	13	3.3	7.9	10	35	7.4	17	8.6	2.1	<0.1	16	3.6	<0.1	1.7	94	93	95
SakSTAR(E80A,D82A)		130	7.3	12	2.1	6.5	5.9	79	6.1	8.4	7.8	1.9	<0.1	<0.1	<0.1	<0.1	0.4	89	83	92
SakSTAR(L81A)	23	28	12	33	1.6	40	11	52	11	17	17	3.9	1.4	5.2	7.1	4.6	1.5	88	95	95
SakSTAR(D82A)		160	17	12	4.8	7.3	11	31	7.8	17	12	2.7	<0.1	0.2	<0.1	<0.1	2.3	95	93	95
SakSTAR(D82A,S84A)	72	130	8.3	14	2.6	8.1	8.5	23	3.8	12	11	1.7	<0.1	<0.1	1.4	<0.1	1.0	91	91	95
SakSTAR(S84A)	12/26	89	8.0	16	3.8	8.6	10	90	8.3	11	36	1.8	2.2	1.6	3.0	3.5	0.5	95	95	95
SakSTAR(K86A,E88A)		73	[7.2	1.4	3.7	6.0	4.6	5.7	4.9	7.7	15	4.4	<0.1	5.4	0.80	1.9	0.13			
SakSTAR(I87A)	18	98	6.7	23	2.8	8.6	9.1	10	3.6	11	7.4	2.7	1.1	7.8	3.4	4.5	1.0	95	95	95
SakSTAR(V89A)	20	87	4.6	11	2.6	6.6	2.2	28	7.2	7.3	3.0	1.3	1.2	5.1	2.9	3.1	0.83	95	95	95
SakSTAR(T90A)	78	120	6.0	12	0.9	3.7	3.1	20	4.8	7.2	<0.1	<0.1	2.1	6.6	2.6	2.1	0.5	95	95	95
SakSTAR(Y91A)	5	53	6.0	16	3.0	7.0	13	28	8.2	16	0.6	2.1	1.4	3.7	1.6	1.6	0.2	95	95	95
SakSTAR(Y92A)	16	120	16	23	4.1	13	12	29	7.3	18	<0.1	1.7	4.4	10	3.9	5.9	1.1	95	95	95
SakSTAR(E93A,K94A)		97	[8.2	19	13	30	24	18	11	>10	9.0	0.88	1.4	11	2.4	7.0	2.1]			
SakSTAR(K94A,N95A,K97A)	32	8	NT															95	94	95
SakSTAR(N95A)	25	260	10	18	4.0	10	11	50	13	14	4.9	2.3	3.7	7.3	4.7	2.9	0.8	95	94	95
SakSTAR(K96A,K97A,K98A)		47	[2.8	41	23	37	90	>16	9.1	19	16	0.41	0.58	17	1.2	13	0.30]			
SakSTAR(E99A)	24	42	7.4	15	4.0	8.4	8.9	22	2.7	4.7	<0.1	<0.1	2.1	6.2	7.3	14	0.8	92	91	92
SakSTAR(E99A,E100A)	LE																			

Table 4: Mutagenesis of S34, G36 and H43: Association constants (K_A x 10^7mol/L^{-1}) for binding to insolubilized murine monoclonal antibodies (Mab) and absorption (percent) of antibodies of immunized patient plasma

nt	Exp. (mg/L)	Spec. Act. (kU/mg)	murine MAbs: Epitope cluster I 17G11	26A2	30A2	2B12	3G10	Epitope cluster II 18F12	14H5	28H4	32B2	7F10	Epitope cluster III 7H11	25E1	40C8	24C4	1A10	SakSTAR patient plasma: Pool	Subpool B	Subpool C
TAR		120	9.3	13	2.9	7.8	11	38	7.4	19	7.7	2.4	4.0	14	5.4	2.9	0.6	95	95	95
SakSTAR(S34G,G36R,H43R)		120	10	14	3.3	7.5	11	<0.1	<0.1	<0.1	20	2.7	<0.1	<0.1	<0.1	0.15	1.7	87	76	75
SakSTAR(S34A)	29	110	17	24	4.6	9.5	11	28	11	22	15	2.9	3.1	8.8	3.8	2.0	0.2	95	95	93
SakSTAR(G36A)	14	72	3.5	9.8	1.5	5.7	6.5	52	4.2	17	9.2	1.4	<0.1	<0.1	0.9	5.0	1.0	86	83	78
SakSTAR(G36E)	12	66	3.1	8.7	1.4	4.8	4.7	12	2.8	6.1	7.6	1.0	<0.1	<0.1	<0.1	3.4	1.1	89	83	72
SakSTAR(G36K)	34	88	9.9	23	3.1	8.3	9.8	21	3.9	13	15	3.0	<0.1	<0.1	<0.1	2.6	1.2	88	80	69
SakSTAR(G36L)	15	92	3.6	11	1.8	6.1	6.1	16	1.4	6.4	12	1.3	<0.1	<0.1	<0.1	0.6	1.1	93	85	72
SakSTAR(G36N)	10	91	8.7	10	1.6	6.1	6.2	33	3.3	7.8	7.9	1.8	<0.1	<0.1	1.0	0.3	0.5	86	80	75
SakSTAR(G36Q)	21	92	10	12	1.8	6.7	6.5	23	3.8	7.5	7.3	1.5	<0.1	<0.1	<0.1	0.1	0.4	87	84	73
SakSTAR(G36R)	45	100	11	2.4	3.3	10	10	27	4.6	14	20	3.4	<0.1	<0.1	<0.1	3.1	1.2	89	81	70
SakSTAR(H43A)	37	69	15	28	9.7	18	7.6	<0.1	<0.1	<0.1	9.1	1.5	2.0	23	7.8	7.2	1.6	95	95	95
SakSTAR(H43R)	43	120	15	11	2.7	7.6	11	<0.1	<0.1	<0.1	13	6.4	0.7	18	6.7	5.7	1.4	95	95	95
SakSTAR(S34G,G36R)	45	90	3.1	12	2.3	4.8	4.2	13	8.3	24	9.1	1.9	<0.1	<0.1	<0.1	<0.1	0.6	92	83	69
SakSTAR(S34G,G36R,H43R,K74A)	1	12	12	4.7	3.8	7.4	9.4	<0.1	0.1	0.6	25	2.3	<0.1	<0.1	0.5	0.8	1.7	67	56	83
SakSTAR(S34G,G36R,K74A)	15	26	4.0	2.1	<0.1	1.8	0.6	10	2.2	15	13	1.8	<0.1	<0.1	<0.1	0.3	2.2	59	28	68
SakSTAR(K35G,G36R,H43D)	32	6	1.8	1.5	1.6	2.0	8.9	<0.1	<0.1	<0.1	7.1	1.3	<0.1	<0.1	<0.1	<0.1	0.9	82	75	72
SakSTAR(G36R,K74A)	40	35	19	7.0	0.2	4.3	2.0	53	27	28	19	4.4	<0.1	<0.1	<0.1	1.2	1.0	48	33	58
SakSTAR(G36R,K74R)	68	150	4.7	17	3.8	11	8.0	16	6.0	6.4	3.0	1.6	<0.1	<0.1	<0.1	0.2	0.8	81	54	73
SakSTAR(G36R,K74A,N95A)	11	25	6.1	2.9	2.9	2.4	<0.1	31	5.7	12	5.3	4.1	<0.1	<0.1	<0.1	<0.1	0.9	53	32	63
SakSTAR(G36R,K74A,K135R)	20	33	5.8	3.4	<0.1	1.7	0.7	26	16	14	17	1.2	<0.1	<0.1	<0.1	0.4	0.5	64	32	68
SakSTAR(G36R,K74R,K135R)	48	75	6.1	17	5.8	10	3.3	31	14	13	5.7	2.3	<0.1	<0.1	<0.1	0.3	0.8	77	49	68

Table 5 - cont'd: Mutagenesis of K35, Y73, K74, E80/D82, N95, K130, V132 and K135: Association constants (K_A x $10^7 mol/L^{-1}$) for binding to insolubilized murine monoclonal antibodies (Mab) and absorption (percent) of antibodies of immunized patient plasma

			murine MAbs															SakSTAR patient plasma		
			Epitope cluster I					Epitope cluster II					Epitope cluster III							
	Exp. (mg/L)	Spec. Act. (kU/mg)	17G11	26A2	30A2	2B12	3G10	18F12	14H5	28H4	32B2	7F10	7H11	25E1	40C8	24C4	1A10	Pool	Subpool B	Subpool C
SakSTAR(K130A)		280	5.1	12	3.2	6.4	3.5	22	6.7	11	15	2.7	<0.1	<0.1	4.1	0.9	0.6	92	74	71
SakSTAR(K130T)		290	7.8	14	3.1	8.0	5.6	43	5.4	9.7	17	2.7	<0.1	<0.1	3.4	0.5	0.5	92	84	82
SakSTAR(V132A)	102	130	4.2	15	2.6	9.2	11	33	12	30	19	2.1	2.1	3.6	<0.1	2.6	0.4	95	95	95
SakSTAR(V132L)	126	120	4.8	14	2.3	8.0	9.7	67	10	48	20	2.5	2.0	11	<0.1	4.8	0.4	95	95	95
SakSTAR(V132T)	78	140	4.5	13	2.4	7.8	9.0	39	10	25	16	2.0	1.1	2.0	<0.1	<0.1	0.4	95	95	95
SakSTAR(V132N)	16	150	4.5	11	1.7	7.0	7.2	21	17	20	15	2.0	1.5	1.3	<0.1	4.2	0.7	95	95	95
SakSTAR(V132R)	76	230	5.4	12	0.8	3.3	3.2	23	5.5	7.8	4.8	1.1	2.1	3.4	<0.1	0.4	0.4	95	95	95
SakSTAR(K135A)	54	410	5.2	12	11	7.9	11	20	11	11	3.8	2.0	1.6	6.9	3.7	1.9	0.9	95	95	95
SakSTAR(K135F)	68	64	3.9	6.3	1.0	6.1	4.1	13	4.5	8.4	16	2.1	1.6	5.8	1.9	1.5	0.9	95	95	95
SakSTAR(K135R)	54	230	4.0	14	1.4	9.3	5.0	31	8.1	13	23	1.9	2.4	2.4	2.6	2.1	0.5	95	95	95
SakSTAR(K35A,K74A)	20	130	NT															84	50	95
SakSTAR(Y73A,K74A)	24	<5	18	<0.1	<0.1	<0.1	<0.1	19	6.7	23	9.9	3.2	2.7	13	4.0	1.6	1.1	47	28	87
SakSTAR(Y73F,K74A)	21	6	17	6.7	<0.1	2.1	<0.1	42	19	21	20	4.9	1.9	2.8	6.6	2.1	0.8	51	34	90
SakSTAR(I60A,K74A,N95A)		84	5.3	2.7	<0.1	2.5	5.3	17	7.2	9.6	3.4	2.3	2.1	6.2	2.7	2.9	0.8	60	47	95
SakSTAR(N95A,K135R)	120	240	4.9	13	1.9	9.0	9.9	18	12	15	2.5	1.5	1.8	5.9	3.6	3.7	0.8	95	95	95
SakSTAR(K130T,K135R)	15	280	3.7	10	1.6	7.2	8.0	13	7.7	4.5	3.7	1.6	<0.1	<0.1	2.4	0.4	0.6	89	60	73

ble 6 - cont'd: Combination mutants of SakSTAR(K130T,K135R) with K35A, G36R, E65X,K74X and selected other amino acids

ant	Exp. mg/L	Spec. Act. (kU/mg)	murine MAbs: Epitope cluster I 17G11	26A2	30A2	2B12	3G10	Epitope cluster II 18F12	14H5	28H4	32B2	7F10	Epitope cluster III 7H11	25E1	40C8	24C4	1A10	SakSTAR patient plasma: Pool 10	Subpool B	Subpool C	Pool 40	Code
TAR(E65Q,K74Q,N95A,K130A,K135R)	40	220	6.1	14	1.9	17	15	9.0	3.3	5.8	6.3	2.3	<0.1	<0.1	4.1	2.2	0.5	52	29	74	58	SY72
TAR(E65Q,K74Q,E118A,K130A,K135R)	86	180	8.5	18	2.8	15	27	11	4.1	5.7	7.3	2.6	<0.1	<0.1	6.1	2.8	0.5	50	28	72	58	SY73
SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R)	33	190	7.8	18	2.4	7.7	21	20	3.9	6.1	6.6	2.3	<0.1	<0.1	5.8	2.5	0.5	48	27	74	58	SY74
SakSTAR(N95A,K130A,K135R)	85	410	6.1	11	3.3	18	15	37	5.9	9.6	6.8	2.5	<0.1	<0.1	4.5	3.0	0.6	93	81	82	94	INT1
SakSTAR(K35A,E65Q,K74Q,K130A,K135R)	29	110	NT															49	26	63	45	SY75
SakSTAR(K35A,H43R,E65Q,K74Q,K130A,K135R)		14	NT															49	23	73	55	SY76
SakSTAR(E65Q,K74Q,S103A,K130A,K135R)	32	60	6.7	15	2.6	14	16	8.0	2.7	3.9	6.3	2.3	<0.1	<0.1	4.6	1.6	0.6	55	27	75	61	SY77
SakSTAR(T21A,K35A,E65Q,K74Q,K130A,K135R)		110	NT															50	26	72	50	SY78
SakSTAR(T56A,E65Q,K74Q,K130T,K135R)		180	NT															52	31	61	55	SY79
SakSTAR(K57A,E58A,E61A,K74Q,K130T,K135R)		120	NT															57	24	61	54	SY80
SakSTAR(E65Q,K74Q,K109A,K130T,K135R)	40	210	7.3	15	2.1	12	12	14	2.5	4.0	5.8	2.3	<0.1	<0.1	3.4	1.8	0.7	50	22	68	51	SY81
SakSTAR(E65Q,K74Q,E108A,K130T,K135R)		120																51	24	61	54	SY82
SakSTAR(E65Q,K74Q,E108A,K109A,K130T,K135R)	62	180	9.3	13	1.4	13	17	17	3.0	4.1	6.8	2.5	<0.1	<0.1	3.7	2.6	0.5	55	21	67	50	SY83
SakSTAR(E65Q,K74Q,K121A,K130T,K135R)	73	150	5.7	13	1.5	11	14	22	3.1	4.6	1.2	<0.1	<0.1	<0.1	3.5	1.8	0.9	61	25	69	57	SY85
SakSTAR(E19A,E65Q,K74Q,K130T,K135R)		3	NT															51	27	62	56	SY86
SakSTAR(E65Q,K74Q,D115A,K130T,K135R)		57	NT															52	25	62		SY87
SakSTAR(G36R,E65A,K74Q,K130E,V132R,K135R)	48	60	7.6	9.9	1.4	11	14	42	19	17	4.3	1.0	<0.1	<0.1	<0.1	<0.1	0.9	44	17	70	44	SY60
SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,+137A)		120																45	30	74	60	SY93
SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,K136A,+137K)		1,400																37	16	70	54	SY94

Association constants ≥ 10-fold lower and antibody absorption ≤60 percent of wild-type SakSTAR are represented in bold type; ≥ 100,000 HU/mg represented in bold type. NT: not tested.

Table 7 - cont'd: Combination mutants of SakSTAR(E80A,D82A,K130T,K135R) with K35A, G36R, E65X, K74X, and selected other amino acids

	Exp. (mg/mL)	Spec. Act. (kU/mg)	murine MAbs: Epitope cluster I 17G11	26A2	30A2	2B12	3G10	Epitope cluster II 18F12	14H5	28H4	32B2	7F10	Epitope cluster III 7H11	25E1	40C8	24C4	1A10	SakSTAR patient plasma: Pool 10	Subpool B	Subpool C	Pool 40	Code
TAR(K74R,E80A,D82A,S103A,K130T,K135R)	32	160	4.9	22	5.8	14	4.3	26	6.9	7.4	5.0	1.7	<0.1	<0.1	<0.1	<0.1	0.9	67	32	69	70	SY24
TAR(K35A,E65D,K74R,E80A,D82A,E108A,K109A,K130T,K135R)	9.0	89	5.8	30	2.6	26	10	13	16	13	3.2	1.8	<0.1	<0.1	<0.1	<0.1	0.5	55	10	63	47	SY12
SakSTAR(K35A,E65D,K74R,E80A,D82A,E108A,K130T,K135R)	20	91	6.4	20	5.0	15	3.9	22	17	7.4	2.4	1.9	<0.1	<0.1	<0.1	<0.1	0.9	44	8	70	53	SY32
SakSTAR(E65D,K74R,E80A,D82A,E108A,K130T,K135R)	4	90	8.1	6.7	6.9	4.3	29	19	31	11	14	2.1	<0.1	<0.1	<0.1	<0.1	1.0	52	11	69	-	SY33
SakSTAR(K35A,E65D,K74R,E80A,D82A,K109A,K130T,K135R)	42	84	5.5	18	5.3	14	1.6	18	12	7.7	10	1.7	<0.1	<0.1	<0.1	<0.1	1.0	43	6	61	50	SY36
SakSTAR(E65D,K74R,E80A,D82A,K109A,K130T,K135R)	60	130	9.7	6.6	6.8	4.2	28	11	32	12	17	2.3	<0.1	<0.1	<0.1	<0.1	0.9	56	10	64	53	SY37
SakSTAR(K35A,E65D,K74R,E80A,D82A,K130T,K135R,K136A)	28	81	4.5	12	3.3	11	1.7	22	13	7.6	4.9	1.6	<0.1	<0.1	<0.1	<0.1	0.8	40	14	52	40	SY34
SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R,K136A)	60	100	6.8	5.8	4.4	4.5	15	33	32	14	7.9	2.0	<0.1	<0.1	<0.1	<0.1	0.8	46	28	67	45	SY35
SakSTAR(E65Q,K74Q,D82A,S84A,K130T,K135R)		170	NT															45	21	60	45	SY50N
SakSTAR(K35A,E65D,K74R,E80A,D82A,K86A,K130T,K135T)	68	86	4.4	20	5.5	15	1.5	15	12	6.4	6.7	1.9	<0.1	<0.1	<0.1	<0.1	1.0	36	7	60	55	SY40
SakSTAR(K35A,K74Q,E80A,D82A,K130T,K135R)	72	120	6.1	3.4	2.5	3.0	5.9	38	14	9.8	6.8	1.9	<0.1	<0.1	<0.1	<0.1	0.8	49	16	64	48	SY28
SakSTAR(K35A,E65D,K74R,E80A,D82A,K130T,K135R)	54	190	8.1	7.5	6.9	5.5	25	37	34	14	7.7	2.3	<0.1	<0.1	<0.1	<0.1	1.0	56	28	68	55	SY29
SakSTAR(K35A,E65D,K74R,E80A,D82A,V132R,K135R)	13	55	6.7	23	5.3	17	2.3	47	19	19	5.1	2.0	<0.1	<0.1	<0.1	<0.1	1.1	53	20	88	62	SY61
SakSTAR(K35A,E65D,K74R,E80A,D82A,T129A,K135R)	13	61	7.0	13	5.1	31	12	27	12	11	6.7	2.5	<0.1	<0.1	<0.1	<0.1	1.9	56	18	79	60	SY62
SakSTAR(K35A,E65D,K74R,E80A,D82A,T129A,K135A)	23	21	6.9	27	5.8	32	20	29	6.6	9.7	5.4	2.1	<0.1	<0.1	<0.1	<0.1	0.9	56	17	91	60	SY64

Association constants ≥ 10-fold lower and antibody absorption ≤60 percent of wild-type SakSTAR are represented in bold type; ≥ 100,000 HU/mg represented in bold type. NT: not tested.

Table 8 - cont'd: SakSTAR variants with intact specific activity (≥ 100 kHU/mg) and ≤50 percent absorption of human antibodies elicited by treatment with wild-type SakSTAR

Variant	Spec. Act. (kU/mg)	SakSTAR patient plasma				Code
		Pool 10	Subpool B	Subpool C	Pool 40	
SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,+137A)	**120**	**45**	**30**	74	**60**	SY93
SakSTAR(E65Q,K74Q,N95A,E118A,K130A,K135R,K136A,+137K)	**1,400**	**37**	**16**	70	**54**	SY94
SakSTAR(E65Q,K74Q,D82A,S84A,E108A,K109A,K130A,K135R)	**110**	**46**	**26**	63	**41**	SY95

Antibody absorption ≤60 percent of wild-type SakSTAR are represented in bold type; ≥ 100,000 HU/mg represented in bold type.

Table 10: Pharmacokinetic parameters of the disposition of staphylokinase-related antigen from plasma following bolus injection of SakSTAR variants (100 μg/kg) in hamsters.

Variant	C_0 (μg/mL)	A (μg/mL)	B (μg/mL)	t1/2 (α) (min)	t1/2 (β) (min)	V_C (mL)	AUC ($\mu g.min.mL^{-1}$)	Cl_p ($mL.min^{-1}$)
SakSTAR	0.8 ± 0.1	0.6 ± 0.1	0.2 ± 0.0	2.8	7.0	13 ± 1.0	4.6 ± 0.4	2.2 ± 0.2
SakSTAR(K74Q,E80A,D82A,K130T,K135R)	0.5 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	2.0	10	20 ± 2.2	2.5 ± 0.3	4.1 ± 0.5
SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R)	0.6 ± 0.0	0.5 ± 0.0	0.1 ± 0.0	2.0	10	16 ± 1.1	2.8 ± 0.2	3.7 ± 0.3
SakSTAR(K35A,E65DK74Q,E80A,D82A,K130T,K135R)	1.1 ± 0.1	1.0 ± 0.1	0.1 ± 0.0	2.0	24	9.6 ± 0.7	6.4 ± 0.5	1.6 ± 0.1

Data are mean ± SEM of 4 experiments.

Table 12: Absorption with SakSTAR variants of antibodies elicited with SakSTAR variants in patients with peripheral arterial occlusion

Treatment	Absorbant	Insolubilized compound: SakSTAR	SakSTAR(K74Q,E80A,D82A,K130T,K135R)	SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R)
SakSTAR (Pool 40)				
	SakSTAR	95		
	SakSTAR(K74Q,E80A,D82A,K130T,K135R)	48		
	SalSTAR(E65D,K74R,E80A,D82A,K130T,K135R)	57		
SakSTAR(K74Q,E80A,D82A,K130T,K135R) (Imb., Vin., Ver., Gie.)				
	SakSTAR	94	95	95
	SakSTAR(K74Q,E80A,D82A,K130T,K135R)	91	93	89
	SalSTAR(E65D,K74R,E80A,D82A,K130T,K135R)	92	94	94
SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) (Urb.)				
	SakSTAR	90	88	85
	SakSTAR(K74Q,E80A,D82A,K130T,K135R)	94	95	94
	SalSTAR(E65D,K74R,E80A,D82A,K130T,K135R)	94	95	94

Data represent median values of the percent absorption with 250 nM absorbant, measured by residual binding to insolubilized compound.

* p= ..... versus SakSTAR; ** p= ..... versus SakSTAR(K74Q,E80A,D82A,K130T,K135R)); and p= ..... versus SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) by paired non-parametric test.

APPENDIX

Table 14: Cysteine substitution variants of SakSTAR

Variant	Spec. Act. (kU/mg)	SakSTAR patient plasma				Code
		Pool 10	Subpool B	Subpool C	Pool 40	
SakSTAR(K96C)	**790**	95	95	95		
SakSTAR(K102C)	**280**	95	95	95		
SakSTAR(K109C)	**1,900**	95	95	94	90	
SakSTAR(K35C)						
SakSTAR(K74C)						
SakSTAR(.....)						
SakSTAR(K35A,E65D,K74Q,E80A,D82A,K109C,K130T,K135R)	**600**	**46**	**32**	**56**	**36**	SY100
SakSTAR(E65Q,K74Q,D82A,S84A,K109C,K130T,K135R)	**1,100**	**54**	**53**	77	**41**	SY102

Antibody absorption ≤60 percent of wild-type SakSTAR are represented in bold type; ≥ 100,000 HU/mg represented in bold type.

CLAIMS

1. Staphylokinase derivatives showing a reduced immunogenicity as compared to wild-type staphylokinase, after administration to patients with arterial thrombosis.

2. Staphylokinase derivatives as claimed in claim 1 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by another amino acid thus reducing the reactivity with a panel of murine monoclonal antibodies.

3. Staphylokinase derivatives as claimed in claim 1 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by another amino acid thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase.

4. Staphylokinase derivatives as claimed in claim 1 having essentially the amino acid sequence as depicted in figure 1 in which one or more amino acids have been replaced by other amino acids, without reducing the specific activity by more than 50 percent.

5. Staphylokinase derivatives SakSTAR(K35X,G36X,E65X,K74X,E80X,D82X,K102X, E108X,K109X,K121X,K130X, K135X,K136X,+137X) having the amino acid sequence as depicted in figure 1 in which the amino acids Lys in position 35, Gly in position 36, Glu in position 65, Lys in position 74, Glu in position 80, Asp in position 82, Lys in position 102, Glu in position 108, Lys in position 109, Lys in position 121, Lys in position 130, Lys in position 135 and/or Lys in position 136 have been replaced with other amino acids and/or in which one amino acid has been added at the COOH-terminus, thus altering the immunogenicity after administration in patients, without markedly reducing the specific activity.

6. Staphylokinase derivatives listed in Tables 1,3,4,5,6,7 and 8, having the amino acid sequence as depicted in figure 1 in which the indicated amino acids have been replaced by

ABSTRACT OF THE DISCLOSURE

Methods for the identification, production and use of staphylokinase derivatives characterized by a reduced immunogenicity after administration in patients. The derivatives of the invention are obtained by preparing a DNA fragment comprising at least the part of the coding sequence of staphylokinase that provides for its biological activity; performing *in vitro* site-directed mutagenesis on the DNA fragment to replace one or more codons for wild-type amino acids by a codon for another amino acid; cloning the mutated DNA fragment in a suitable vector; transforming or transfecting a suitable host cell with the vector; culturing the host cell under conditions suitable for expressing the DNA fragment; and purifying the expressed staphylokinase derivative to homogeneity. Preferably the DNA fragment is a 453 bp *Eco*RI-*Hind*III fragment of the plasmid *pMEX602sakB*, (*pMEX.SakSTAR*), the *in vitro* site-directed mutagenesis is performed by spliced overlap extension polymerase chain reaction and the mutated DNA fragment is expressed in *E. coli* strain TG1 or WK6. The invention also relates to pharmaceutical compositions comprising at least one of the staphylokinase derivatives according to the invention together with a suitable excipient, for treatment of arterial thrombosis.

1													14
Ser	Ser	Ser	Phe	Asp	Lys	Gly	Lys	Tyr	Lys	Lys	Gly	Asp	Asp

15													28
Ala	Ser	Tyr	Ph		Glu	Pro	Thr	Gly	Pro	Tyr	Leu	Met	Val	Asn

29													42
Val	Thr	Gly	Val	Asp	Ser	Lys	Gly	Asn	Glu	Leu	Leu	Ser	Pro

43													56
His	Tyr	Val	Glu	Phe	Pro	Ile	Lys	Pro	Gly	Thr	Thr	Leu	Thr

57													70
Lys	Glu	Lys	Ile	Glu	Tyr	Tyr	Val	Glu	Trp	Ala	Leu	Asp	Ala

71													84
Thr	Ala	Tyr	Lys	Glu	Phe	Arg	Val	Val	Glu	Leu	Asp	Pro	Ser

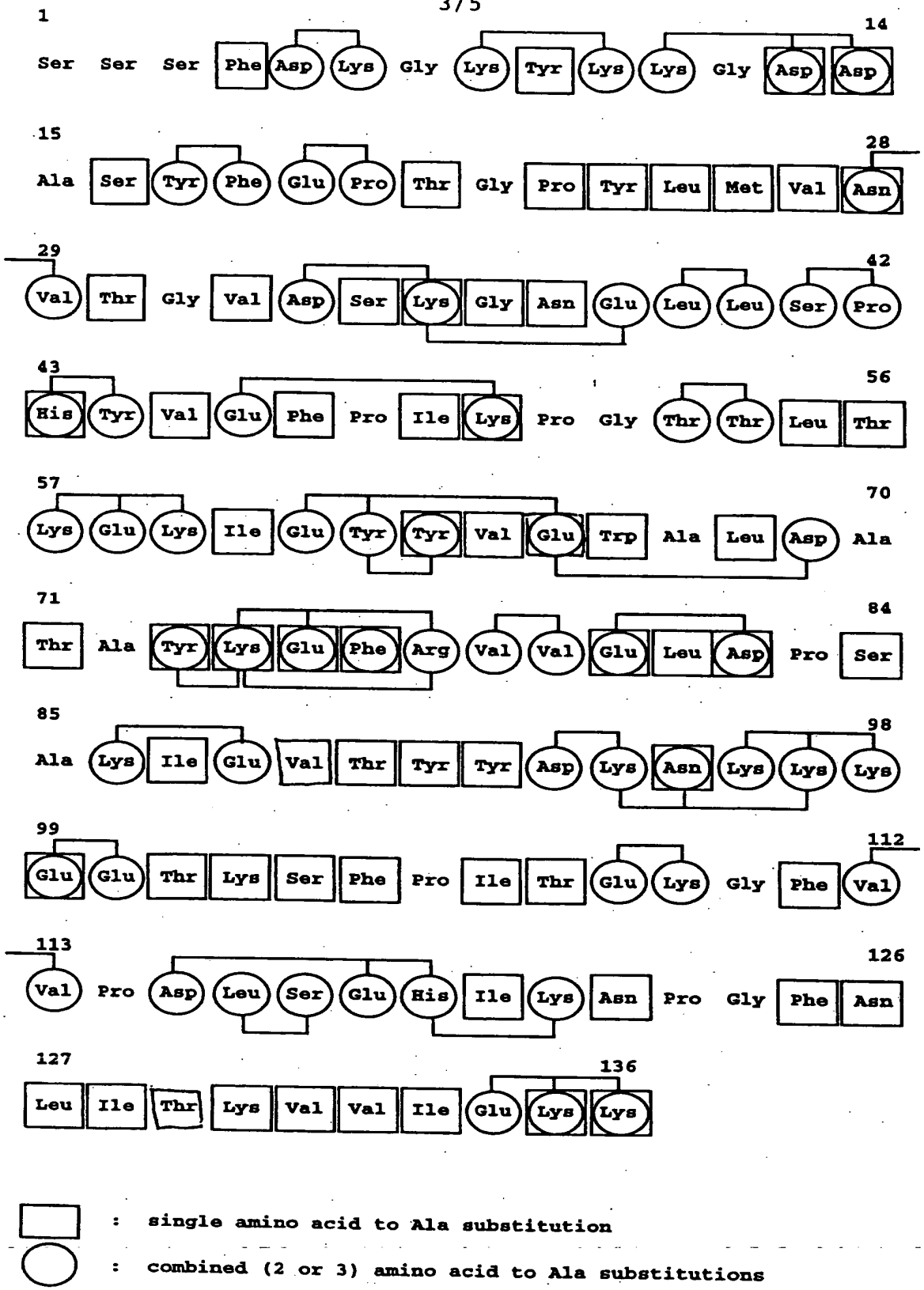
85													98
Ala	Lys	Ile	Glu	Val	Thr	Tyr	Tyr	Asp	Lys	Asn	Lys	Lys	Lys

99													112
Glu	Glu	Thr	Lys	Ser	Phe	Pro	Ile	Thr	Glu	Lys	Gly	Phe	Val

113													126
Val	Pro	Asp	Leu	Ser	Glu	His	Ile	Lys	Asn	Pro	Gly	Phe	Asn

127									136
Leu	Ile	Thr	Lys	Val	Val	Ile	Glu	Lys	Lys

Figure 1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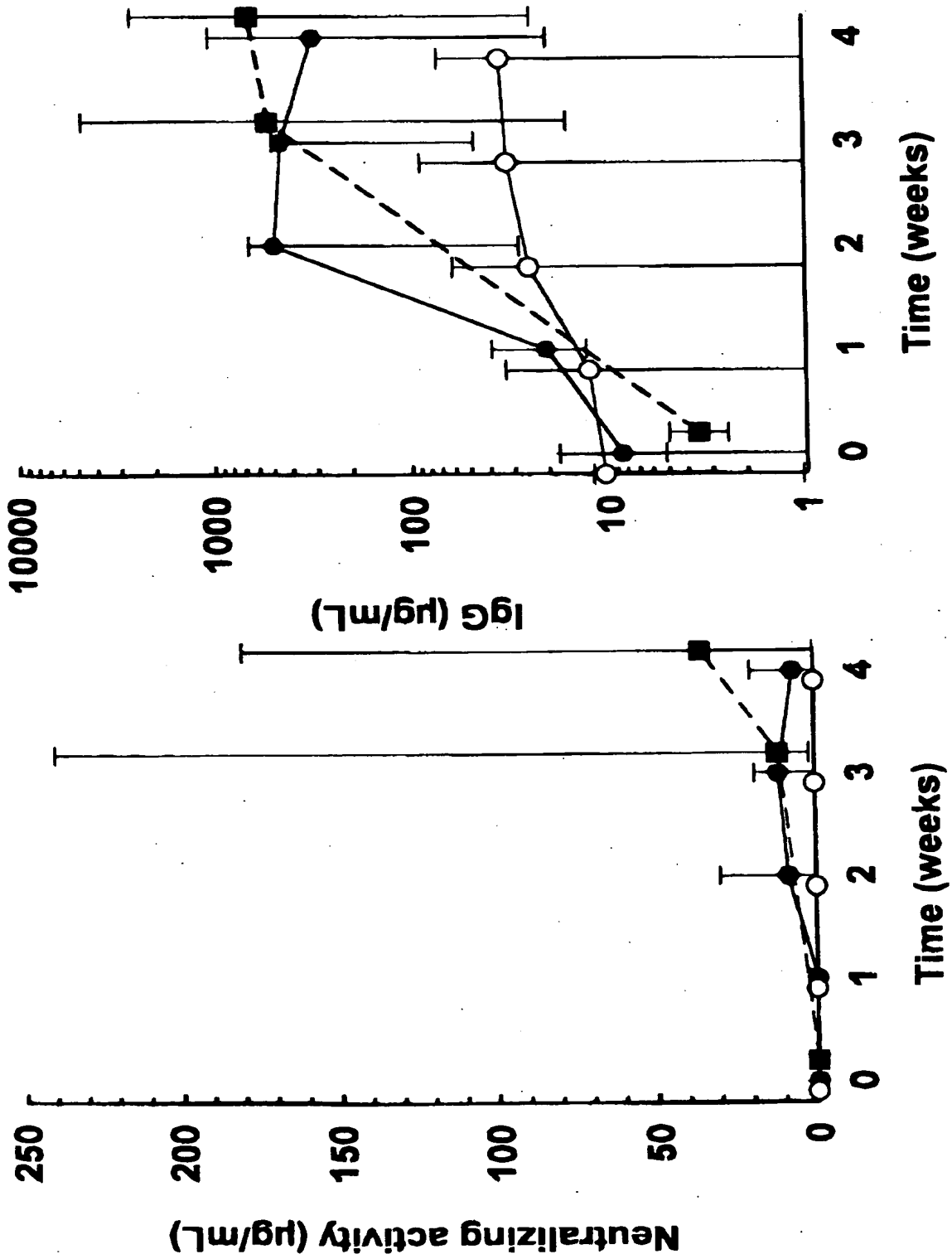
42
43
His Tyr Val Glu Phe Pro Ile Lys Pro Gly Thr Thr Leu Thr
56
57
Lys Glu Lys Ile Glu Tyr Tyr Val Glu Trp Ala Leu Asp Ala
70
71
Thr Ala Tyr Lys Glu Phe Arg Val Val Glu Leu Asp Pro Ser
84
85
Ala Lys Ile Glu Val Thr Tyr Tyr Asp Lys Asn Lys Lys Lys
98
99
Glu Glu Thr Lys Ser Phe Pro Ile Thr Glu Lys Gly Phe Val
112
113
Val Pro Asp Leu Ser Glu His Ile Lys Asn Pro Gly Phe Asn
126
127
Leu Ile Thr Lys Val Val Ile Glu Lys Lys
136
: single amino acid to Ala substitution
: combined (2 or 3) amino acid to Ala substitutions

Figure 3

Figure 5


IgG (µg/mL)
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1000
100
10
1
0
1
2
3
4
Time (weeks)
Neutralizing activity (µg/mL)
250
200
150
100
50
0
0
1
2
3
4
Time (weeks)